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(51) International Patent Classification ⁶ : A61K 35/14, 38/16	A1	(11) International Publication Number: WO 99/18979 (43) International Publication Date: 22 April 1999 (22.04.99)
(21) International Application Number: PCT/US98/19768 (22) International Filing Date: 22 September 1998 (22.09.98) (30) Priority Data: 60/061,663 10 October 1997 (10.10.97) US (71) Applicant: BAXTER INTERNATIONAL INC. [US/US]; One Baxter Parkway, Deerfield, IL 60015 (US). (72) Inventors: HAI, Ton, That; 812 Buckingham Court, Mundelein, IL 60060 (US). NELSON, Deanna, J.; 201 Pond Ridge Road, Libertyville, IL 60040 (US). PEREIRA, David, E.; 6217 Hidden Oak Drive, Crystal Lake, IL 60014 (US).		(81) Designated States: AU, CA, JP, Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
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(54) Title: STORAGE-STABLE HEMOGLOBIN COMPOSITION (57) Abstract A hemoglobin composition including deoxyhemoglobin, oxyhemoglobin in an amount less than about 10 % based upon the total hemoglobin content of the composition, and a dithiol or a disulfide in an amount effective to reduce oxyhemoglobin to deoxyhemoglobin.		

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STORAGE-STABLE HEMOGLOBIN COMPOSITION

Background of the Invention

The present invention relates to a storage-stable hemoglobin composition and a method for inhibiting degradation of a hemoglobin composition during storage. More specifically, the present invention relates to a hemoglobin composition that can
5 be stored at room temperature while remaining physiologically active.

Hemoglobin solutions are known to lose their ability to function as blood substitutes during storage. To improve shelf life, the loss of function is generally delayed by refrigerating or freezing the solutions, or controlling the oxygenation state of the hemoglobin within the solution.

10 During storage, a hemoglobin solution loses its ability to function as a blood substitute because of spontaneous transformation of oxyhemoglobin in the solution to methemoglobin. Methemoglobin is physiologically inactive — it does not bind oxygen and does not function as a blood substitute by releasing oxygen into a patient's bloodstream. Methemoglobin is formed by an autoxidation reaction in
15 which an electron is transferred from a heme-bound ferrous atom (Fe^{2+}) to bound oxygen to provide hemoglobin having a ferric atom (Fe^{3+}) and a superoxide radical anion (Winterbourn et al., "Reactions Involving Superoxide and Normal and Unstable Hemoglobins," Biochem. J., 155:493-502, 1976; Watkins et al., "Autoxidation Reactions of Hemoglobin A Free from Other Red Cell Components: A Minimal
20 Mechanism," Biochem. Biophys. Res. Commun., 132:742-748, 1985).

Autoxidation of oxyhemoglobin is known to occur more rapidly when the hemoglobin solution also contains deoxyhemoglobin (Balagopalakrishna et al., "Production of Superoxide from Hemoglobin-Bound Oxygen Under Hypoxic Conditions," Biochem., 35:6393-6398, 1996). Oxygen within the solution binds to
25 deoxyhemoglobin to form oxyhemoglobin, which is then autoxidized to methemoglobin. Such solutions have been reported to be unfit for therapeutic use after prolonged storage in oxygen-permeable containers at 4°C (Menu et al., "Usual Physicochemical Criteria Provide Insufficient Evidence that a Functional Hemoglobin Solution Can Be Used for Transfusions After Storage for 36 Months at +4°C,"
30 Biomat., Art. Cells, Art. Org., 18(2):169-181, 1990).

Alternatively, hemoglobin solutions have been stored in a completely deoxygenated state to render them stable to degradation (Dilorio, "Preparation of Derivatives of Ferrous and Ferric Hemoglobin," Methods in Immunology, Academic Press, New York, 76:57-72, 1981; Antonini and Brunori, Hemoglobin and Myoglobin in Their Reactions With Ligands, North Holland Publishing Company, Amsterdam, pp. 13-71, 1971). In practice, completely deoxygenating a hemoglobin solution has been commercially impractical. Instead, a hemoglobin solution is typically deoxygenated until the oxyhemoglobin content is about 2-5%, and the partially oxygenated solution is either used or stored (U.S. Patent No. 5,352,773).

Autoxidation is also accelerated when the hemoglobin solution is stored at room temperature; its shelf life is generally less than two or three days (DeVenuto, "Stability of Hemoglobin Solution During Storage," J. Lab. Clin. Med., 92(6):946-952, 1978). Hemoglobin solutions that were stored at room temperature did not function as a blood substitute (Kramlovà et al., "Stroma-Free Hemoglobin Solution for Infusion: Changes During Storage," Haematologia, 10(3-4):365-371, 1976; DeVenuto, "Stability of Hemoglobin Solution During Storage," J. Lab. Clin. Med., 92(6):946-952, 1978). Such limited room-temperature shelf life has led the blood substitutes industry to fully oxygenate therapeutic hemoglobin solutions, freeze or refrigerate the solutions in oxygen-permeable, plastic solution bags to slow the rate of methemoglobin formation during storage, and thaw to room temperature before use.

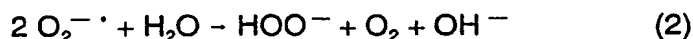
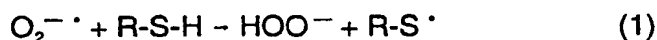
Room-temperature storage is most desirable so that hemoglobin solutions can be immediately administered to patients. Immediate use can be a life-saving measure in emergency medical situations such as trauma, stroke, shock and cardiac arrest. Stability during room-temperature storage also prevents mistakes in handling the solution such as inadvertent or prolonged unrefrigeration or inappropriate thawing which may cause the hemoglobin to become physiologically inactive.

U.S. Patent No. 5,352,773 describes a storage method that enables storage of a therapeutic hemoglobin solution at room temperature by filling an oxygen-impermeable plastic container with the solution in a low oxygen environment and storing the solution in the container. Initially, oxyhemoglobin in the solution autoxidizes to methemoglobin, oxygen binds to deoxyhemoglobin to form oxyhemoglobin for autoxidation, and the methemoglobin content of the solution increases. When the oxyhemoglobin in the solution is nearly depleted, the

methemoglobin spontaneously reduces to deoxyhemoglobin and the methemoglobin content of the solution decreases during storage to less than 50% and preferably less than 15% methemoglobin. After this autoreduction of methemoglobin, the solution is stored for a prolonged time at a temperature between -270°C and 45°C before being administered to a patient as a blood substitute.

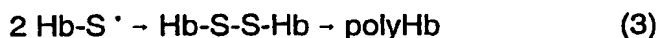
The mechanism for the autoreduction of methemoglobin under anaerobic conditions is unknown, but may result from reduction of methemoglobin to deoxyhemoglobin by the thiol groups present on hemoglobin itself. It is well known that methemoglobin is reduced very slowly by thiols such as cysteine under anaerobic conditions via a mechanism involving a thiyl radical. A thiyl radical may react with a globin or a porphyrin ligand to generate a thiol-modified globin or a sulfhemoglobin, respectively. The modified globin may degrade, denature, or increase the immunogenicity of the hemoglobin composition (Riechlin, Adv. Immunol., 220:71-132, 1975). Although a sulfhemoglobin can bind and release oxygen, it does so at rates that may be a hundred-fold or more slower than those of native hemoglobin.

When oxyhemoglobin is converted to methemoglobin, a generated superoxide anion can decrease the stability of hemoglobin. The superoxide anion can be quenched with a thiol or dismutated according to reaction schemes (1) and (2), respectively:



The peroxide anion (HOO^-) can react catalytically with iron to generate a hydroxyl radical. The oxygen generated in scheme (2) can bind to hemoglobin and continue such undesirable radical chain reactions.

Hemoglobin having at least one thiol group can also self-polymerize, generating hemoglobin disulfides and polymers of hemoglobin covalently joined by disulfide bonds, as shown in the following reaction scheme:



The hemoglobin disulfides are susceptible to oxidation to methemoglobin and polymerization, and the polymers are susceptible to denaturation and precipitation. Thus, in addition to loss of function due to autooxidation of oxyhemoglobin to methemoglobin, therapeutic hemoglobin solutions may degrade, denature or form

precipitates during storage because of hemoglobin self-polymerization and other undesirable hemoglobin reactions.

It has been suggested that the addition of an anti-oxidant or reducing agent to a hemoglobin solution will improve storage stability. However, not all reducing agents can reduce hemoglobin and some may even act as hemoglobin oxidants. Moreover, some anti-oxidants or reducing agents are unsuitable for therapeutic use, may be effective only within a pH range that is not tolerated well physiologically, or are toxic to mammals in the concentrations required to show effectiveness as an anti-oxidant.

10 ~~WO 96/34889 reports that a hemoglobin composition containing a partially~~
deoxygenated hemoglobin solution (i.e., a solution containing less than 5000 parts per million of oxygen) and less than 4 moles of a reducing agent, such as dithionite, sodium borohydride, ascorbate, ferrous salts, or α -tocopherol, per mole of hemoglobin is storage stable for at least three months when stored at temperatures
15 less than about 40°C. However, typical anti-oxidants simply reduce the extent of hemoglobin oxidation during the oxidative phase but do not eliminate the oxidation process. For example, the addition of N-acetyl-L-cysteine, a recognized anti-oxidant, has been found to reduce the extent of hemoglobin oxidation but does not eliminate the oxidation.

20 A need continues to exist for hemoglobin compositions that can be stored at room temperature and remain physiologically active until use.

Summary of the Invention

Among the objects of the present invention, therefore, may be noted the provision of a hemoglobin composition that is stable after storage at temperatures up
25 to about 40°C; the provision of a hemoglobin composition that remains physiologically active throughout storage and later therapeutic use; and the provision of a hemoglobin composition that can be stored at room temperature so that it is immediately available for use.

30 The present invention is directed to a hemoglobin composition including deoxyhemoglobin, oxyhemoglobin in an amount less than about 10% based upon the total hemoglobin content of the composition, and a dithiol or a disulfide in an amount effective to reduce the oxyhemoglobin to deoxyhemoglobin.

The present invention is also directed to a hemoglobin composition containing between about 80% and 100% deoxyhemoglobin, up to about 10% oxyhemoglobin, and at least about 0.1 mole of a dithiol or a disulfide per mole of hemoglobin in the composition.

- 5 Another embodiment of the invention is a process for preparing a hemoglobin composition by providing a hemoglobin composition containing deoxyhemoglobin and up to about 10% oxyhemoglobin based upon the total hemoglobin content of the composition, and admixing a dithiol or a disulfide with the hemoglobin composition.

Yet another embodiment of the invention is directed to a method for inhibiting
10 degradation of a hemoglobin composition during storage by storing a hemoglobin composition containing deoxyhemoglobin and a dithiol or a disulfide in an oxygen-impermeable container at a temperature less than about 40°C.

Brief Description of the Drawings

- 15 FIG. 1 is a plot of the methemoglobin content of partially oxygenated hemoglobin solutions as a function of time during storage at room temperature wherein -Δ- represents the control DCLHb[®] solution, -■- represents a solution having an N-acetyl-L-cysteine (NAC) to DCLHb[®] molar ratio of 1:1, and -O- represents a solution having an NAC/DCLHb[®] molar ratio of 2:1;

- 20 FIG. 2 is a plot of the methemoglobin content of partially oxygenated hemoglobin solutions as a function of time during storage at room temperature wherein -Δ- represents the control DCLHb[®] solution, -■- represents a solution having a dihydrolipoic acid (DHLA) to DCLHb[®] molar ratio of 1:1, and -O- represents a solution having a DHLA/DCLHb[®] molar ratio of 2:1; and

- 25 FIG. 3 is a plot of the methemoglobin content of partially oxygenated hemoglobin solutions as a function of time during storage at room temperature wherein -Δ- represents the control DCLHb[®] solution, -■- represents a solution having a DHLA/DCLHb[®] molar ratio of 3:1, and -O- represents a solution having a DHLA/DCLHb[®] molar ratio of 4:1.

Detailed Description of the Preferred Embodiments

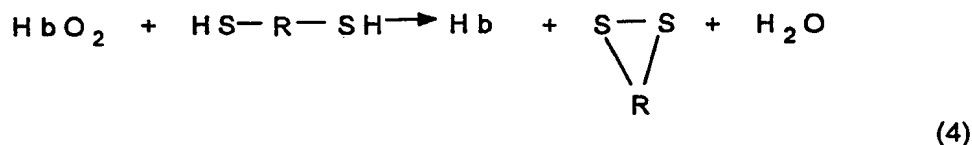
- 30 In accordance with the present invention, it has been discovered that the addition of a dithiol or disulfide to a partially oxygenated hemoglobin composition reduces oxyhemoglobin to deoxyhemoglobin to deplete oxyhemoglobin in the

composition. The reduction reaction maintains the biological activity of the composition by eliminating the autoxidation of oxyhemoglobin to physiologically-inactive methemoglobin. It has also been discovered that the addition of an excess of the dithiol or disulfide accelerates the rate of autoreduction of methemoglobin to deoxyhemoglobin, and can also minimize or prevent hemoglobin self-polymerization to significantly reduce formation of precipitates in the hemoglobin composition during storage. Thus, it has been found that a deoxygenated hemoglobin composition or a partially oxygenated hemoglobin composition remains therapeutically useful throughout storage at temperatures up to about 40°C if a dithiol or disulfide is added to the composition and the composition is stored in an oxygen-impermeable container.

The hemoglobin composition of the invention contains deoxyhemoglobin and a dithiol, a disulfide, or a mixture of a dithiol and a disulfide. If the composition is partially oxygenated when it is manufactured (i.e., it includes oxyhemoglobin in an amount less than about 10% oxyhemoglobin based upon the total hemoglobin content of the composition, preferably less than about 5%, and more preferably, less than about 2%), the dithiol or disulfide or mixture thereof is present in an amount sufficient to reduce oxyhemoglobin in the composition to deoxyhemoglobin and thereby eliminate any autoxidation of oxyhemoglobin to methemoglobin. The dissolved oxygen content of the hemoglobin composition is preferably less than about 10 ppm, more preferably less than about 1 ppm, and most preferably less than about 0.150 ppm.

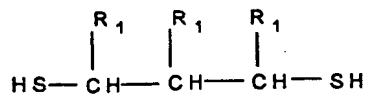
The composition can contain an excess of a dithiol, a disulfide, or a mixture of a dithiol and a disulfide to accelerate the rate at which methemoglobin in the composition is reduced to deoxyhemoglobin and to decrease degradation of the composition during storage so that the hemoglobin composition remains useful for its intended purpose. For example, if the composition is to be used as a blood substitute, the dithiol or disulfide is present in an amount sufficient to decrease degradation during storage so that the composition maintains its ability to transport and deliver oxygen to cells. The term "degradation" includes any measurable indication of hemoglobin deterioration such as oxidation to methemoglobin, precipitate formation, and less readily measured hemoglobin degradation such as undesirable hemoglobin polymerization, undesirable globin modification, and sulfhemoglobin formation.

While not wishing to be bound by any particular theory, it is believed that oxyhemoglobin is reduced by a dithiol, for example, to provide deoxyhemoglobin, a cyclic disulfide and water as shown in the reaction scheme below:



5 Reduction of methemoglobin in the hemoglobin composition then proceeds once oxyhemoglobin is eliminated. Residual dithiol or disulfide in the composition provides sacrificial thiol that is consumed by reaction before thiol within hemoglobin reduces and hemoglobin self-polymerized as shown in reaction scheme (3). Thus, the residual dithiol or disulfide minimizes and may prevent undesirable
10 polymerization and precipitation.

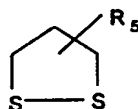
The hemoglobin composition contains between about 0.1 mole and about 10 moles of dithiol per mole of hemoglobin in the composition, preferably between about 0.2 moles and about 5 moles of dithiol per mole of hemoglobin, more preferably between about 0.2 moles and about 2 moles of dithiol per mole of
15 hemoglobin, and most preferably, between about 0.2 moles and about 1 mole of dithiol per mole of hemoglobin. The dithiol preferably has the formula:



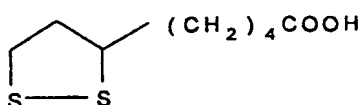
wherein two of the R_1 groups are hydrogen and the remaining R_1 group is $-(\text{CH}_2)_n-\text{C}(\text{O})\text{OR}_2$, $-(\text{CH}_2)_n-\text{C}(\text{O})\text{NR}_3\text{R}_4$, or $-(\text{CH}_2)_n-\text{C}(\text{O})\text{O}^-$ or a salt thereof, n is an integer
20 from 0 to 12, R_2 is hydrogen or alkyl, R_3 is hydrogen or alkyl, and R_4 is hydrogen or alkyl. A particularly preferred dithiol is dihydrolipoic acid, which has the formula $\text{HS}-\text{CH}_2-\text{CH}_2-\text{CH}((\text{CH}_2)_4\text{COOH})\text{SH}$, or its salts.

The hemoglobin composition contains between about 0.1 mole to about 10 moles of disulfide per mole of hemoglobin in the composition, preferably between
25 about 0.5 mole and about 6 moles of disulfide per mole of hemoglobin, more preferably between about 0.5 mole and about 2 moles of disulfide per mole of

hemoglobin, and most preferably, between about 0.6 mole and about 1.5 moles of disulfide per mole of hemoglobin. The disulfide preferably has the formula:



wherein R_5 is $-(CH_2)_n-C(O)OR_2$, $-(CH_2)_n-C(O)NR_3R_4$, or $-(CH_2)_n-C(O)O^-$ or a salt thereof, n is an integer from 0 to 12, R_2 is hydrogen or alkyl, R_3 is hydrogen or alkyl, and R_4 is hydrogen or alkyl. A particularly preferred disulfide is lipoic acid, which has the formula



, or its salts.

The hemoglobin composition is prepared by deoxygenating the composition until a desired oxyhemoglobin content is obtained, and adding a dithiol or disulfide to the composition. The composition is deoxygenated by any known method such as gas-gas separation, gas-liquid separation or sorption processes, and is then maintained in an oxygen-free atmosphere throughout its preparation. The oxyhemoglobin content of the hemoglobin composition can be determined in any known manner such as by spectroscopy or by use of a device for measuring dissolved oxygen content in solution (e.g., a MOCON analyzer as manufactured by Mocon, Minneapolis, MN).

Once the oxyhemoglobin content of the composition is decreased to less than about 10%, a dithiol or disulfide is added to the hemoglobin composition in an amount at least sufficient to reduce the oxyhemoglobin present in the composition to deoxyhemoglobin. An excess of the dithiol or disulfide can be added to increase the rate of methemoglobin reduction in the hemoglobin composition.

After the addition of the dithiol or disulfide, the composition is maintained in an inert atmosphere (i.e., an atmosphere free of oxygen) by any known method, such as by packaging the composition in an oxygen-impermeable container as described in U.S. Patent No. 5,352,773. If desired, the hemoglobin compositions of

the invention can be stored at temperatures less than about 40°C for periods of several months or longer.

In a preferred embodiment, the hemoglobin composition is a pharmacologically acceptable solution to which is slowly added an aqueous solution of a dithiol or disulfide which has been degassed using nitrogen or argon. The solution is stirred after addition to ensure homogeneity, and then apportioned into oxygen-impermeable containers for storage.

The compositions may be formulated for subcutaneous, intravenous, intra-arterial, intra-peritoneal or intramuscular injection or infusion, arterial cannulization, or topical or oral administration in small or large volumes to mammals such as humans. Therapeutic hemoglobin compositions are preferably administered systemically. The compositions can be administered in a single dose, or in a series of multiple subdoses. The single dose or each of the multiple subdoses can be administered by slow continuous infusion.

The hemoglobin compositions of the present invention can be prepared for many prophylactic or therapeutic uses in both clinical and veterinary medicine. Such uses include, but are not limited to, use as a blood substitute for oxygen delivery or volume expansion; use in treating and/or preventing and/or reducing the severity of anemia, angina, ischemia, myocardial infarction, cerebral infarction, shock such as hemorrhagic, septic, anaphylactic, allergic, burn or cardiogenic shock, stroke, subarachnoid hemorrhage, cerebral vasospasms, thalassemia, cardiac arrest, cytopenia, cachexia, sepsis, hemorrhage, hypotension, edema, congestive heart failure, sickling crisis, reperfusion injury, stenosis, restenosis, respiratory disease, schistosomiasis, schizophrenia, contusions, Alzheimer's disease, amyotrophic lateral sclerosis, malaria, muscular dystrophies, muscle contraction, fatigue or spasm, depression, diabetes, gastric mucosa, head injury, hyperemia, hyperlipidemia, hypoxia, infection, thrombosis or other occlusions, diarrhea, Parkinson's disease, gastrointestinal disorders, carbon monoxide poisoning, or colic; use in stimulating hematopoiesis, hemodialysis, angioplasty, cardiopulmonary resuscitation, binding or delivery of nitric oxide or non-oxygen ligands, cell proliferation, imaging and other diagnostic methods, wound healing, oxygen extraction, hemodilution, hemoaugmentation, cardiopulmonary bypass, drug delivery, calibration, or pathenogenic fungi control; use as an oxidizing agent, cell culture media, antilipidemic agent, carrier, reference solution, cyanide poison antidote, vasodilator,

surgical pretreatment, vasoconstrictor, trace metal binder, extracorporeal pump primer, tissue or organ perfusing agent, or iron source; or in conjunction with chemotherapy or radiation cancer treatments.

For most uses, the methemoglobin content of the hemoglobin composition is preferably less than 50%, more preferably less than 30%, and most preferably less than 15% based on the total hemoglobin content of the composition. However, some uses such as treatment of carbon monoxide poisoning, may necessitate greater methemoglobin content.

The hemoglobin composition is administered in dosages between about 10 milligrams and about 20 grams or more per kilogram of body weight over a period of seconds to hours. The dosage, period of administration, and manner of dosing the patient depend upon the condition being treated, the form of the hemoglobin composition, the condition of the patient, and other factors from which one of ordinary skill in the medical arts can determine an appropriate dosage.

As used herein, the term "hemoglobin" includes all proteins containing globin or globin-like polypeptides and heme. The term "hemoglobin" includes all naturally- and non-naturally-occurring hemoglobin. The term "hemoglobin preparation" includes hemoglobin, which is capable of transporting and releasing oxygen to cells, tissues or organs when introduced into the blood stream of a mammal, in a physiologically compatible carrier or as lyophilized and reconstituted with a physiologically compatible carrier, but does not include whole blood, red blood cells or packed red blood cells. The term "hemoglobin composition" does not include whole blood, red blood cells, or packed red blood cells. The term "deoxyhemoglobin" includes all deoxygenated hemoglobin, and the term "oxyhemoglobin" includes all oxygenated hemoglobin. The term "methemoglobin" includes all hemoglobin containing iron in the ferric (Fe^{3+}) state.

Naturally-occurring hemoglobin includes any hemoglobin identical to hemoglobin naturally existing within a cell. Naturally-occurring hemoglobin is predominantly wild-type hemoglobin, but also includes naturally-occurring mutant hemoglobin. Wild-type hemoglobin is hemoglobin most commonly found within natural cells. Wild-type human hemoglobin includes hemoglobin A, the normal adult human hemoglobin having two α - and two β -globin chains. Mutant hemoglobin has an amino-acid sequence that differs from the amino-acid sequence of wild-type hemoglobin as a result of a mutation, such as a substitution, addition or deletion of at

least one amino acid. Adult human mutant hemoglobin has an amino-acid sequence that differs from the amino-acid sequence of hemoglobin A. Naturally-occurring mutant hemoglobin has an amino-acid sequence that has not been modified by humans. The naturally-occurring hemoglobin of the present invention is not limited
5 by the methods by which it is produced. Such methods typically include, for example, erythrocytolysis and purification, recombinant production, and protein synthesis.

Non-naturally-occurring hemoglobin includes mutant hemoglobin having an amino-acid sequence different from the amino-acid sequence of hemoglobin
10 ~~naturally existing within a cell, and chemically-modified hemoglobin.~~ Such non-naturally-occurring mutant hemoglobin is not limited by its method of preparation, but is typically produced using one or more of several techniques known in the art, including, for example, recombinant DNA technology, transgenic DNA technology, protein synthesis, and other mutation-inducing methods.

15 Chemically-modified hemoglobin is a natural or non-natural hemoglobin molecule which is bonded to or encapsulated by another chemical moiety. For example, a hemoglobin molecule can be bonded to pyridoxal-5'-phosphate, or other oxygen-affinity-modifying moiety to change the oxygen-binding characteristics of the hemoglobin molecule, to crosslinking agents to form crosslinked or polymerized
20 hemoglobin, or to conjugating agents to form conjugated hemoglobin. Conjugated, polymerized and crosslinked hemoglobins generally exhibit longer intravascular retention times than unmodified hemoglobin.

Several examples of hemoglobin modification technology which can be used in the practice of the present invention have been described in the scientific literature
25 (reviewed by R. M. Winslow (1992) in *Hemoglobin-Based Red Cell Substitutes*, The Johns Hopkins University Press, Baltimore, MD). Some representative methods of preparing chemically-modified hemoglobin for use in the invention are described below.

Hemoglobin can be modified to improve its oxygen-binding affinity. Reagents
30 that bind to the 2,3-diphosphoglycerate binding site of a hemoglobin molecule, reduce the oxygen affinity of the hemoglobin molecule, and prolong intravascular retention are described in U.S. Patent Nos. 4,529,719 and 5,380,824 (pyridoxal-5'-phosphate), U.S. Patent No. 4,600,531 (carboxyl-, phosphonate-, phosphate-, sulfonate- or sulfate-phenyl ester-containing compounds such as mono(3,5-

dibromosalicyl)fumarate), U.S. Patent No. 5,268,500 (aryluroid acid compound), U.S. Patent No. 5,382,680 (2[4-(((benzyl)amino)carbonyl) phenoxy]-2-methyl propionic acids), and U.S. Patent Nos. 5,290,803 and 5,432,191. In general, any method of preparing or modifying hemoglobin such that the hemoglobin can transport and release oxygen is suitable in the present method. Preferably, the hemoglobin has a P_{50} of between about 20 and about 45 mm Hg.

An encapsulated hemoglobin is hemoglobin surrounded by a material which retains the hemoglobin within the material yet allows smaller molecules to pass through the material to react with hemoglobin and reaction products to pass out of the material. Materials for encapsulating hemoglobin are described in U.S. Patent No. 4,343,715 (polyurethane, acrylic gels, maleic anhydride polymers, epoxy polymers, glutaric aldehyde polymers), U.S. Patent Nos. 5,061,688, 5,217,648 and 5,438,041 (oil emulsion), U.S. Patent No. 4,911,929 (liposomes), and U.S. Patent Nos. 4,322,311, 4,324,683 and 4,390,521 (polymers).

A conjugated hemoglobin is at least one non-hemoglobin molecule covalently or ionically bound to a hemoglobin. In some embodiments, the non-hemoglobin molecule can also form an intermolecular crosslink between hemoglobin molecules. Conjugating materials and methods for preparing hemoglobin conjugates are described in WO 91/07190 (polyalkylene glycol), U.S. Patent Nos. 4,670,417, 5,091,176, 5,219,564, 5,234,903, 5,312,808 and 5,386,014, WO 94/04193, WO 94/09027 and Japanese Patent Nos. 59-104323 and 61-053223 (polyalkylene oxide), U.S. Patent Nos. 5,349,001 and 5,405,877 (cyclic imide thione activated polyalkylene oxide), U.S. Patent No. 4,301,144 (polyalkylene glycol, alkylene glycol copolymers, alcohol-polyalkylene glycol ether copolymers, carboxylic acid-polyalkylene glycol ester copolymers, and amine-polyalkylene glycol derivatives), U.S. Patent Nos. 4,267,234, 4,267,435 and 4,369,226 (polyglutaraldehyde), Canadian Patent Application No. 2,074,852 (divinyl sulfone), U.S. Patent No. 4,412,989 (polyether), U.S. Patent No. 4,377,512 (inulin), U.S. Patent Nos. 5,079,337 and 5,110,909 (polysaccharide, polyvinyl alcohol, polyvinyl pyrrolidone, polymethacrylate, polypeptide, polyalkylene glycol, hydroxyalkyl starch, and dextran), U.S. Patent No. 4,920,194 (sulfated glycosaminoglycan fragments, such as heparin), U.S. Patent No. 4,970,156 (active protein), U.S. Patent No. 4,336,248 (dialdehyde), U.S. Patent No. 4,900,780 (hydroxyethyl starch or tetronic polymer), and U.S. Patent Nos. 4,698,387, 4,935,465, and 5,514,780.

Crosslinked hemoglobin is intramolecularly linked between globin or globin-like protein subunits by a crosslinking agent. A subunit is one globin or globin-like protein of a hemoglobin molecule. Intramolecular crosslinking prevents dissociation of globin or globin-like proteins when hemoglobin is administered *in vivo*.

- 5 Hemoglobin A, for example, can dissociate into two α - β globin dimers if the dimers are not crosslinked. Crosslinked hemoglobins and methods for their preparation are described in U.S. Patent Nos. 4,529,719 and 4,600,531 (α - α linkage using diphenyl ester derivatives such as bis(3,5-dibromosalicyl)fumarate), U.S. Patent Nos. 4,001,401 and 4,053,590 (α - β globin linkage using halogenated cycloalkanes, 10 diepoxides, and diazobenzidines), U.S. Patent No. 4,857,636 (aldehyde derived from oligosaccharide), U.S. Patent No. 5,334,705 (benzenetricarboxylate), WO 94/21682 (β - β globin linkage using di- or trisaccharide), U.S. Patent No. 5,290,919 and 5,387,672 (di- or trivalent compounds), U.S. Patent No. 5,334,707 (β - β or α - α linkage using acyl phosphate ester), U.S. Patent No. 5,362,885 and WO 92/09630 15 (imidoesters, such as dimethyl adipimidate or dimethyl suberimidate), U.S. Patent No. 5,514,780 (polycarboxylic acid), U.S. Patent No. 5,399,671 and WO 90/13309 (β - β linkage), and U.S. Patent No. 4,473,496 (dialdehyde).

- A polymerized hemoglobin is intermolecularly linked between hemoglobin molecules. Polymerization generally increases the molecular weight of the 20 hemoglobin, which improves its intravascular half-life. Polymerization agents for preparing polymerized hemoglobin are described in pending U.S. applications Serial Nos. 08/149,679, 08/173,882, 08/480,593, and 08/473,459, U.S. Patent No. 4,777,244 (aliphatic dialdehyde), U.S. Patent No. 5,349,054 (benzenepentacarboxylate), WO 94/14460 (transglutaminase), and EP 201618 25 (glutaraldehyde).

- Hemoglobins can also be modified by a combination of the methods described above, for example, as described in Japanese Patent Nos. 59-089629, 59-103322, and 59-104323 (pyridoxal-5'-phosphate modification and polyethylene glycol conjugation of hemoglobin), U.S. Patent No. 5,248,766 (crosslinking and 30 polymerization of tetrameric hemoglobins with oxiranes), U.S. Patent Nos. 4,650,786, 4,710,488 and 4,900,816 (inositol phosphate aldehyde modification and polysaccharide conjugation of hemoglobin), U.S. Patent Nos. 5,189,146 and 5,364,932 (di- or polyaldehydes for intra- and intermolecular crosslinking), EP 361719 (pyridoxylation, dicarboxylic acid halo-ester crosslinking, and

polymerization), WO 90/13309 (pyridoxal-5-phosphate derivative for intramolecular crosslinking and glutaraldehyde for polymerization), U.S. Patent No. 5,439,882 (periodate-oxidized ATP intramolecular crosslinking and periodate-oxidized adenosine polymerization), U.S. Patent Nos. 4,826,811 and 5,194,590
5 (pyridoxylation and glutaraldehyde polymerization), and U.S. Patent No. 4,529,719 (intramolecularly crosslinked with diaspirin ester and pyridoxylated).

Recombinantly-produced hemoglobin is produced by recombinant DNA methodologies, for example, by site-directed mutagenesis, gene fusion, or transfecting a genetically engineered plasmid into a microorganism such as a
10 bacterium or yeast, a cultured cell such as an insect cell, a mammalian cell, or plant cell, a transgenic plant, a transgenic animal, or any other host cell or organism, where the plasmid includes a nucleic acid polymer (e.g., cDNA) which encodes a globin protein, a fusion protein, or a protein similar to globin that can reversibly bind oxygen. Recombinant mutant and artificial hemoglobins and their production in cell
15 cultures or fluids is described in U.S. Patent Nos. 5,776,890, 5,661,124, 5,563,254, 5,449,759 and 5,028,588, and in WO 88/09179, AU 614525, GB 2234749 B, and EP 358708 B1. Di- α and di- β globin-like polypeptides and other hemoglobin variants produced in bacteria and yeast, and other fused hemoglobins, are described in U.S. Patent Nos. 5,801,019, 5,798,227, 5,744,329, 5,679,777, and 5,545,727, and in WO
20 90/13645, WO 91/16349, WO 93/08842, EP 402300 B1, EP 561245 A1, EP 611306 B1, EP 700997 A1, AU 665599, AU 635744, and AU 614525. Non-natural multimeric hemoglobin-like proteins are described in U.S. Patent Nos. 5,739,011 and 5,599,907, and in EP 611376 B1, AU 672960, WO 93/09143 and WO 96/40920. Hemoglobin variants containing non-naturally occurring binding domains are
25 described in WO 97/23631. Production and recovery of human hemoglobin from transgenic pigs are described in WO 92/22646, WO 93/25071, and WO 95/04744. Methods for the preparation and purification of hemoglobin derived from erythrocyte and non-erythrocyte cells are described in WO 92/22646, WO 93/25071, WO
95/04744, WO 95/14038, and WO 96/15151.

30 Hemoglobins useful in the compositions of the present invention are also free of pyrogens, toxins and other contaminants. Pyrogen-free hemoglobin is hemoglobin that is absolutely free of fever-producing contaminants, or hemoglobin that contains amounts of fever-producing contaminants that are physiologically acceptable to patients to which the hemoglobin will be administered. Bacterial

endotoxins can contaminate hemoglobin derived from erythrocytes. Recombinant hemoglobin produced in non-erythrocyte host cells such as bacteria can also become contaminated with cellular components such as proteins, toxins, or polysaccharides that can elicit toxic or pyrogenic responses when administered to mammals (Rietschel et al. (1992) *Scientific American* 267:54-61; Suffredini et al. (1989) *New Eng. J. Med.* 321:280-287).

Hemoglobins for use in the present invention are also stroma-free. Stroma, the insoluble cell membrane fragments that contaminate hemoglobin derived from lysed erythrocytes, is toxic and has been reported to cause dyspnea, bronchospasm, hypotension, arrhythmia, disseminated intravascular coagulation, activation of complement, and renal, myocardial, and hepatic changes associated with ischemia and acute inflammation (Feola (1988) *Surgery, Gynecology & Obstetrics* 166:211-222; MacDonald et al. (1988) *F.A.S.E.B. J.* 2(6) Abstr. 8217; Stone et al. (1979) *Surgery, Gynecology & Obstetrics* 149:874-876; Rabiner et al. (1967) *J. Exp. Med.* 126:1127-1142. For purposes of the present invention, "stroma-free hemoglobin" is hemoglobin, as defined herein, which is either absolutely free of stroma, or which contains stroma at concentrations that are physiologically acceptable (i.e., do not cause adverse side effects) in a patient. Stroma-free hemoglobin that is absolutely free of stroma includes recombinant hemoglobin produced in a non-erythrocyte. Stroma-free hemoglobin that contains stroma at physiologically acceptable levels includes, for example, purified hemoglobin derived from erythrocytes.

The hemoglobin can be dialyzed or exchanged by ultrafiltration into a physiologically acceptable solution preferably to between about 1 and about 20 g/dl hemoglobin. The solution generally comprises a physiologically compatible electrolyte vehicle isosmotic with whole blood and which maintains the reversible oxygen-carrying and delivery properties of the hemoglobin. The physiologically acceptable solution can be, for example, physiological saline, a saline-glucose mixture, Ringer's solution, lactated Ringer's solution, Locke-Ringer's solution, Krebs-Ringer's solution, Hartmann's balanced saline, heparinized sodium citrate-citric acid-dextrose solution, and polymeric plasma substitutes, such as polyethylene oxide, polyvinyl pyrrolidone, polyvinyl alcohol and ethylene oxide-propylene glycol condensates. Such solutions can be administered parenterally, for example by intravenous or intra-arterial injection or infusion (i.e., systemic administration), without adverse side effects. The hemoglobin can also be lyophilized for storage

and reconstituted prior to use. Methods for preparing such solutions or lyophilized powders are known in the art. The composition can include other components such as salts (e.g., sodium chloride, potassium chloride), buffers (e.g., lactate, gluconate, phosphate), surfactants or chelating agents (e.g., ethylenediamine pentaacetic acid) and the like. The pH of the hemoglobin composition is preferably from about 6 to about 8 at 37°C.

A preferred hemoglobin for use in the present invention is hemoglobin crosslinked with bis(3,5-dibromosalicyl) fumarate to create a fumarate crosslink between the two α subunits (DCLHb[®], manufactured by Baxter Healthcare, Deerfield, Illinois). This crosslinked hemoglobin is more fully described, together with methods for its preparation, in U.S. Patents Nos. 4,598,064, 4,600,531, and RE 34,271, omitting the chromatography step. This hemoglobin is preferably manufactured under the conditions disclosed in U.S. Patent Nos. 4,831,012, 4,861,867, 5,128,452 and 5,281,579 and U.S. patent application serial no. 07/207,346.

In practice, a preferred DCLHb[®] solution, manufactured by Baxter Healthcare Corporation and useful in the present invention, contains 10 g/dl of modified tetrameric hemoglobin in a balanced electrolyte solution. The product is prepared from units of human red cells from volunteer donors which have been tested and found negative for HbsAg, HIV-1 and 2, and HCV. During manufacture, the red cells are osmotically lysed to release hemoglobin. After ultrafiltration, the stroma-free hemoglobin is reacted with the diaspirin crosslinking agent to produce a stabilized tetrameric hemoglobin having a fumaryl moiety linking the two α subunits. After crosslinking, the reaction mixture is heated to effect viral deactivation and precipitate extraneous proteins. The precipitate is removed by filtration. The DCLHb[®] is then concentrated and diafiltered into a physiologic electrolyte vehicle. The resulting solution is isosmotic with whole blood, hyperoncotic (approximately 40 torr), and has the composition shown in Table 1.

TABLE 1

**Chemical Assay of
10% Diaspirin Crosslinked
Hemoglobin Solution**

5

Hemoglobin content	10 g/dl
Oncotic pressure	43 mm Hg
Osmolarity	290 mOsm/L
pH	7.4 @ 37°C
Na ⁺	145 mEq/L
K ⁺	4 mEq/L
Ca ⁺⁺	2.3 mEq/L
Mg ⁺⁺	0.9 mEq/L
Cl ⁻	115 mEq/L
Lactate	34 mEq/L

10

15

The following examples are presented to describe preferred embodiments and utilities of the present invention and are not meant to limit the present invention unless otherwise stated in the claims appended hereto.

EXAMPLE 1

The following deoxyhemoglobin solutions were prepared:

- 20 1. Control solution: DCLHb[®] (1.55 mM) in lactated electrolyte solution, pH 7.5.
2. Control solution + N-Acetyl-L-Cysteine (NAC) in a molar ratio of NAC:DCLHb[®] of 1:1.
3. Control solution + NAC in an NAC:DCLHb[®] molar ratio of 2:1.
4. Control solution + Gluconate (3 mM).
- 25 5. Control solution + Dihydrolipoic Acid (DHLA) in a DHLA:DCLHb[®] molar ratio of 1:1.
6. Control solution + DHLA in a DHLA:DCLHb[®] molar ratio of 2:1.

Each deoxyDCLHb solution was prepared as follows. The DCLHb[®] solution (1.55 mM in lactated electrolyte solution) was deoxygenated at 25°C by passing the
30 solution through a membrane oxygenator charged with oxygen-free nitrogen gas.

Deoxygenation was stopped when the level of oxyhemoglobin was about 10%. The appropriate additive (N-acetylcysteine, gluconate or dihydrolipoic acid) was added to provide solutions 2-6. All manipulations were performed under nitrogen. The solutions were stored at room temperature; in addition, the DHLA solution was kept
5 in the dark. At intervals, each of the deoxyDCLHb solutions was passed through a 0.2 μ pore-size filter, and the filtrate was analyzed by on-line, multi-wavelength, UV-visible spectroscopic analysis (Van Assendelft et al., *Analyt. Biochem.*, 69:43-48, 1975), followed by application of the algebraic formulae for the determination of methemoglobin, oxyhemoglobin, and deoxyhemoglobin percentages and total
10 hemoglobin content.

On day 0, the methemoglobin content of each of the solutions was about 28%. During the next eight days the methemoglobin content of the control solution increased to about 55% methemoglobin and then decreased gradually to about 36% methemoglobin by day 28, as shown in FIGs. 1 and 2.

15 Over a four-day period the test solution containing 3 mM gluconate showed an increase to about 40% methemoglobin and then a gradual decrease to about 36% methemoglobin. Thus, gluconate appeared to prevent hemoglobin oxidation but had no other observable effects.

In the presence of the recognized reducing agent/anti-oxidant NAC (in an
20 NAC:DCLHb[®] molar ratio of 1:1 or 2:1), the methemoglobin content increased by 1-3% during day 1 and then decreased quite rapidly to about 9 and 3% methemoglobin, respectively, as measured on day 28 as illustrated in FIG. 1. Thus, NAC in either molar ratio was capable of minimizing the oxidative phase, but not eliminating it. A higher molar ratio of NAC facilitated the methemoglobin reduction
25 phase.

In contrast, no oxidative phase was observed in the solutions containing 1 or 2 mole equivalents of DHLA as shown in FIG. 2. Within day 1 the methemoglobin content of these test solutions was reduced to about 16% and 5%, respectively. Gradual methemoglobin reduction continued, so that by day 28 the methemoglobin
30 content of these solutions was about 3% and 0%, respectively. Thus, DHLA was the only reducing agent/anti-oxidant studied that eliminated the oxidation phase. Moreover, DHLA was a very effective methemoglobin reducing agent.

The changes in solution turbidity were used as a surrogate monitor for precipitate formation. The turbidity of the solutions was determined using a Hach

2100AN turbidimeter. The turbidity results were reported in Normalized Turbidity Units (NTUs) corresponding to the difference between filtered (0.2 μ pore-size filter) and unfiltered samples. The turbidity measurements are reported in Table 2 below.

TABLE 2

Time Days	Solution Turbidity in Normalized Turbidity Units (NTU's)					
	Control	+1:1 NAC	+2:1 NAC	+ Gluconate	+1:1 DHLLA	+2:1 DHLLA
0	0.5	0.1	9	0.1	0.1	0.3
1	5	25	51	3	6	6
2	20	85	103	12	6	18
3	58	112	134	19	34	23
4	100	140	145	75	59	27
7	188	174	189	120	135	41
9	206	184	213	183	167	39
11	277	212	211	206	163	66
14	278	201	225	212	226	39
17	269	183	203	214	239	43
23	219	155	206	213	282	41
28	312	183	223	196	316	39

The solution turbidity significantly increased from 0 NTU to values ranging from 183-316 NTU's for all deoxyDCLHb solutions except for the solution containing DHLLA in a molar ratio of 2:1, for which an increase in turbidity of only 39 NTU's was observed. These results suggest that the presence of an excess of DHLLA significantly reduces the extent of particulate formation.

The properties of the deoxyDCLHb in solution were assessed by thiol determination, size-exclusion chromatography (SEC), reversed-phase high performance liquid chromatography (RP-HPLC), and SDS-PAGE electrophoresis. SEC was performed using Superdex™ 200 column (Pharmacia), a mobile phase consisting of 50 mM phosphate, pH 7.0, containing 0.15 M NaCl, delivered at a flow

rate of 0.7 mL/min., and analyte detection at 215 nm. In this assay, materials elute from the stationary phase in the order from largest to smallest in size, i.e., larger entities elute with shorter retention times and smaller entities elute with longer retention times. RP-HPLC was performed using a Vydac Protein C-18 column, with elution using mobile phases (A) and (B) delivered at 1 mL/min. as a linear gradient having the following compositions over time: 1) 0% B to 40% B over 60 minutes; 2) 40% B to 78% B over 20 minutes; 3) hold at 78% B for 2 minutes; 4) 78% B to 100% B over 10 minutes. Mobile phase (A) consisted of CH₃CN/H₂O/TFA, 60:40:0.1, by volume. Mobile phase (B) consisted of CH₃CN/H₂O/TFA, 60:40:0.1, by volume.

5
10 Analytes were monitored at 215 nm. SDS-PAGE electrophoresis was completed under reducing and non-reducing conditions. Thiol determination was completed by the Neis method (Neis et al., Toxicology, 31:319-329, 1984).

In general, all of these assays indicated that the structure of the deoxyDCLHb in solution does not change during storage. For example, the number of reactive
15 thiols (2) per DCLHb[®] in the control and the gluconate-containing solutions did not change during storage. When NAC was added to a control solution in a molar ratio of 1:1 or 2:1, the number of reactive thiols per DCLHb[®] decreased from 3 and 4, respectively, to 2 in about seven days and then remained at about 2 until day 28. Similarly, when DHLA was added in a molar ratio of either 1:1 or 2:1, the number of
20 reactive thiols per DCLHb[®] (which were expected to be 4 and 6, respectively, on day 0) were 2 on day 0 and throughout the 28 days of storage. These results indicate that NAC and DHLA act as sacrificial anti-oxidants to prevent the modification of reactive thiols in deoxyDCLHb solutions, and that DHLA is a much stronger anti-oxidant than NAC in this regard.

25 Likewise, the SEC profiles of deoxyDCLHb solutions on day 0 and day 14 were typical of DCLHb[®] and indicate that no soluble polyDCLHb was formed during storage. The RP-HPLC profiles of deoxyDCLHb in solution on day 0 and day 14 also showed no remarkable changes. The ratios of the peak areas of the α - α / β globin subunits of DCLHb[®] were constant for all six deoxyDCLHb solutions during
30 storage, confirming the absence of structural change from degradation in the deoxyDCLHb in solution. Finally, SDS-PAGE analyses of deoxyDCLHb solutions from day 0 and day 28 indicated no significant structural change in the deoxyDCLHb in any of the six solutions during storage.

These results indicate that the deoxyDCLHb that remained in solution did not change structurally during storage at room temperature for 28 days, except for decreases in methemoglobin content. The addition of a recognized anti-oxidant such as NAC reduced the extent of hemoglobin oxidation and accelerated methemoglobin reduction. However, DHLA eliminated the hemoglobin oxidation phase, significantly accelerated methemoglobin reduction, and decreased precipitate formation. The data suggest that excess DHLA may prevent precipitate formation.

EXAMPLE 2

The following deoxyhemoglobin solutions were prepared:

1. Control solution: DCLHb[®] (1.55 mM) in lactated electrolyte solution, pH 7.5.
2. Control solution + DHLA in a DHLA:DCLHb[®] molar ratio of 3:1.
3. Control solution + DHLA in a DHLA:DCLHb[®] molar ratio of 4:1.

The DCLHb[®] control solution (1.55 mM in lactated electrolyte solution) was deoxygenated at 25°C by passing the solution through a membrane oxygenator charged with oxygen-free nitrogen gas. Deoxygenation was stopped when the level of oxyhemoglobin was about 10%. DHLA was added to provide solutions 2 and 3. All manipulations were performed under nitrogen. The solutions were stored in darkness at room temperature. At intervals, each deoxyDCLHb solution was passed through a 0.2 μ pore-size filter, and the filtrate was analyzed to determine methemoglobin content as described in Example 1. The change in methemoglobin content during storage at 25°C is shown in FIG. 3. The small fluctuations in methemoglobin content observed between day 0 and 1 were within the experimental error of the method. DHLA eliminated the hemoglobin oxidation phase and significantly accelerated methemoglobin reduction.

EXAMPLE 3

A DCLHb[®] solution (1 mM in a 0.1 M HEPES solution at pH 8.0) was deoxygenated at 25°C by passing the solution through a membrane oxygenator charged with oxygen-free nitrogen gas. Deoxygenation was stopped when the level of oxyhemoglobin was about 10%. DHLA was added to provide solutions having a molar ratio of DHLA:DCLHb[®] of 0.5:1, 1:1, 1.5:1 and 2:1. All manipulations were performed under nitrogen. The solutions were stored in darkness at room temperature. At intervals, each deoxyDCLHb solution was passed through a 0.2 μ

pore-size filter, and the filtrate was analyzed to determine methemoglobin content as described in Example 1. The change in methemoglobin content during storage under anaerobic conditions at 5°C is shown in Table 3 below.

TABLE 3

Time (Hours)	Molar Ratio of DHLA to DCLHb [®]			
	0.5	1	1.5	2
	Methemoglobin Content (wt.%)			
0	24.5	24.6	24.8	24.5
1	15.4	12.1	8.9	7.9
2	8.9	5.0	2.5	2.2
3	3.3	2.1	NM	NM
4	2.6	2.4	1.3	1.5
5	NM	NM	0.9	1.2
21	3.8	5.0	NM	NM
22	NM	NM	2.7	3.3
24	3.2	4.9	2.3	3.0
26	3.5	5.5	2.4	3.1

NM = not measured

DHLA eliminated the hemoglobin oxidation phase and accelerated the methemoglobin reduction rate more rapidly at greater molar ratios of DHLA:DCLHb[®].

EXAMPLE 4

Lipoic acid (LA) was added to the DCLHb[®] solution of Example 3 to provide solutions having a molar ratio of LA:DCLHb[®] of 0.5:1, 1:1, 1.5:1 and 2:1.

The solutions were stored in darkness at room temperature. At intervals, each deoxyDCLHb solution was passed through a 0.2 μ pore-size filter, and the filtrate was analyzed to determine methemoglobin content as described in Example 1.

The change in methemoglobin content during storage under anaerobic conditions at 5°C is shown in Table 4 below.

TABLE 4

Time (Hours)	Molar Ratio of LA to DCLHb [®]			
	0.5	1	1.5	2
	Methemoglobin Content (wt.%)			
0	12.4	12.1	12.8	12.7
1	12.4	12.1	12.8	12.7
2	12.4	12.1	12.7	12.7
3	12.4	12.2	NM	NM
5	NM	NM	12.6	12.5
22	12.0	11.5	12.1	11.8
24	11.9	11.5	11.9	11.7

NM = not measured

Lipoic acid eliminated the hemoglobin oxidation phase. Methemoglobin reduction proceeded at a slower rate as compared to solution containing DHLA.

EXAMPLE 5

Eight domestic crossbred barrows of 10-14 kg body weight were catheterized surgically and then allowed to stabilize for 24 hours. 20 mL/kg body weight of a 10 g/dl DCLHb[®] in lactated electrolyte solution containing 1.28 g of lipoic acid per liter of DCLHb[®] (LA-DCLHb) was administered to four barrows as a topload infusion at a rate of 1 mL/kg/min. The units of LA-DCLHb were covered with foil during infusion because lipoic acid is photosensitive. 20 mL/kg body weight of a 10 g/dl DCLHb[®] solution was administered to two barrows as a reference control at a rate of 1 mL/kg/min. The two remaining barrows were untreated controls.

Baseline measurements were obtained in each animal prior to treatment, and additional sampling was performed immediately post-treatment and at 0.5, 1,

8, 24 and 48 hours post-infusion. About 48 hours after dosing, the animals were sacrificed and necropsied. The heart, brain, lung, liver, kidneys, spleen, pancreas, and gastrointestinal tract were collected and fixed for histopathological examination by a veterinary pathologist.

5 The physiological parameters monitored included heart rate; mean, systolic and diastolic blood pressures; hematocrit; and plasma hemoglobin and methemoglobin content. The blood chemistries monitored included blood urea nitrogen (BUN), lactate, creatinine, electrolytes, total protein, albumin, non-heme globulin, osmolality, and the enzymes amylase, lipase, alanine aminotransferase
10 (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK)(total and isoenzymes). Hematological parameters monitored included total and differential white blood cell counts; prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, and fibrin degradation products.

15 In general, the effects of the LA-DCLHb were similar to those of the reference control as observed in this study and as reported in previous studies. Increased MAP between 40-50 mm Hg above baseline, increased activities of AST, LDH and CK, minor gastrointestinal distress (emesis and/or diarrhea), and minimal to moderate microscopic lesions of recent myocardial necrosis were
20 observed. The presence of minor unilateral or bilateral, focal cortical and/or proximal tubular necrosis in three of the four LA-DCLHb-treated animals was attributed to the lipoic acid, since these changes were not observed in this study or previous studies in pigs receiving DCLHb® alone. No effects were observed for the other physiological or hematological parameters or blood chemistries
25 monitored.

 Following a review of the data it was concluded that, at the concentration used, lipoic acid did not significantly or adversely affect the biocompatibility of DCLHb®.

EXAMPLE 6

30 Six domestic crossbred barrows of 10-14 kg body weight were catheterized surgically and then allowed to stabilize for 24 hours. 20 mL/kg body weight of a 10 g/dl DCLHb® in lactated electrolyte solution containing 1.28 g of lipoic acid per liter of DCLHb® (LA-DCLHb) were administered to four barrows as

a topload infusion at a rate of 1 mL/kg/min. The units of LA-DCLHb were covered with foil during infusion because lipoic acid is photosensitive. 20 mL/kg body weight of a 10 g/dl deoxyDCLHb solution were administered to one barrow as a reference control at a rate of 1 mL/kg/min. The remaining barrow was an untreated control.

Baseline measurements were obtained in each animal prior to treatment, and additional sampling was performed immediately post-treatment and at 0.5, 1, 8, 24 and 48 hours post-infusion. About 48 hours after dosing, the animals were sacrificed and necropsied. The heart, brain, lung, liver, kidneys, spleen, pancreas, and gastrointestinal tract were collected and fixed for histopathological examination by a veterinary pathologist.

The physiological parameters monitored included heart rate; mean, systolic and diastolic blood pressures; hematocrit; and plasma hemoglobin and methemoglobin content. The blood chemistries monitored included BUN, lactate, creatinine, electrolytes, total protein, albumin, non-heme globulin, osmolality, and the enzymes amylase, lipase, alanine, ALT, AST, LDH, and CK(total and isoenzymes). Hematological parameters monitored included total and differential white blood cell counts, PT, APTT, fibrinogen, and fibrin degradation products.

In general, the effects of the LA-DCLHb were similar to those of the reference control as observed in this study and as reported in previous studies. Increased activities of AST, LDH and CK, minor gastrointestinal distress (emesis and/or diarrhea), and minimal to moderate microscopic lesions of recent myocardial necrosis were observed. The presence of minor unilateral or bilateral, focal cortical and/or proximal tubular necrosis in all of the LA-DCLHb-treated animals was attributed to the lipoic acid, since these changes were not observed in this study or previous studies in pigs receiving DCLHb® alone. No effects were observed for the other physiological or hematological parameters or blood chemistries monitored.

Following a review of the data it was concluded that, at the concentration used, lipoic acid did not significantly or adversely affect the biocompatibility of deoxyDCLHb.

The contents of each of the references cited herein are incorporated herein by reference in their entirety.

While the invention is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of example in the drawings and have been described herein in detail. It should be understood, however, that it is not intended to limit the invention to the particular form disclosed, but on the contrary, the intention is to cover all modifications, 5 equivalents and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

WE CLAIM:

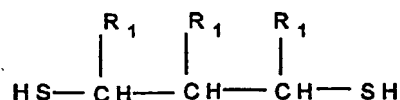
1. A hemoglobin composition comprising deoxyhemoglobin; oxyhemoglobin in an amount less than about 10% based upon the total hemoglobin content of the composition; and a dithiol or a disulfide in an amount effective to reduce the oxyhemoglobin to deoxyhemoglobin.

2. The hemoglobin composition of claim 1 wherein the composition includes between about 0.1 mole and about 10 moles of a dithiol or a disulfide per mole of hemoglobin in the composition.

3. The hemoglobin composition of claim 1 wherein the composition includes between about 0.1 mole and about 6 moles of a dithiol or a disulfide per mole of hemoglobin in the composition.

4. The hemoglobin composition of claim 1 wherein the composition includes between about 0.1 mole and about 2 moles of a dithiol or a disulfide per mole of hemoglobin in the composition.

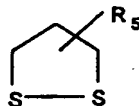
5. The hemoglobin composition of claim 1 wherein the composition contains the dithiol and the dithiol has the formula



wherein two of the R_1 groups are hydrogen and the remaining R_1 group is $-(\text{CH}_2)_n-\text{C}(\text{O})\text{OR}_2$, $-(\text{CH}_2)_n-\text{C}(\text{O})\text{NR}_3\text{R}_4$, or $-(\text{CH}_2)_n-\text{C}(\text{O})\text{O}^-$ or a salt thereof, n is an integer from 0 to 12, R_2 is hydrogen or alkyl, R_3 is hydrogen or alkyl, and R_4 is hydrogen or alkyl.

6. The hemoglobin composition of claim 1 wherein the dithiol is dihydrolipoic acid or a salt thereof.

7. The hemoglobin composition of claim 1 wherein the composition contains the disulfide and the disulfide has the formula



- 5 wherein R_5 is $-(CH_2)_n-C(O)OR_2$, $-(CH_2)_n-C(O)NR_3R_4$, or $-(CH_2)_n-C(O)O^-$ or a salt thereof, n is an integer from 0 to 12, R_2 is hydrogen or alkyl, R_3 is hydrogen or alkyl, and R_4 is hydrogen or alkyl.

8. The hemoglobin composition of claim 1 wherein the disulfide is lipoic acid or a salt thereof.

9. The hemoglobin composition of claim 1 wherein the hemoglobin is selected from the group consisting of crosslinked hemoglobin, conjugated hemoglobin, encapsulated hemoglobin, recombinantly-produced hemoglobin, and polymerized hemoglobin.

10. The hemoglobin composition of claim 9 wherein the crosslinked hemoglobin is diaspirin-crosslinked hemoglobin.

11. The hemoglobin composition of claim 1 wherein the hemoglobin composition is in the form of a physiologically acceptable solution for parenteral administration.

12. The hemoglobin composition of claim 11 wherein the physiologically acceptable solution contains from about 1 g/dl to about 20 g/dl hemoglobin.

13. The hemoglobin composition of claim 1 including no more than about 15% methemoglobin based upon the total hemoglobin content of the composition.

14. A hemoglobin composition comprising between about 80% and 100% deoxyhemoglobin based upon the total hemoglobin content of the composition; up to about 10% oxyhemoglobin based upon the total hemoglobin content of the

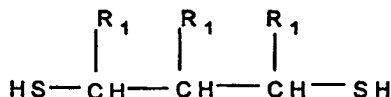
composition; and at least about 0.1 mole of a dithiol or a disulfide per mole of
 5 hemoglobin in the composition.

15. The hemoglobin composition of claim 14 wherein the composition includes between about 0.1 mole and about 10 moles of a dithiol or a disulfide per mole of hemoglobin in the composition.

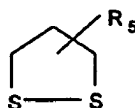
16. The hemoglobin composition of claim 14 wherein the composition includes between about 0.1 mole and about 6 moles of a dithiol or a disulfide per mole of hemoglobin in the composition.

17. The hemoglobin composition of claim 14 wherein the composition includes between about 0.1 mole and about 2 moles of a dithiol or a disulfide per mole of hemoglobin in the composition.

18. The hemoglobin composition of claim 14 including the dithiol having the formula



or the disulfide having the formula



5

wherein two of the R_1 groups are hydrogen, the remaining R_1 group is $-(\text{CH}_2)_n-\text{C}(\text{O})\text{OR}_2$, $-(\text{CH}_2)_n-\text{C}(\text{O})\text{NR}_3\text{R}_4$, or $-(\text{CH}_2)_n-\text{C}(\text{O})\text{O}^-$ or a salt thereof, R_5 is $-(\text{CH}_2)_n-\text{C}(\text{O})\text{OR}_2$, $-(\text{CH}_2)_n-\text{C}(\text{O})\text{NR}_3\text{R}_4$, or $-(\text{CH}_2)_n-\text{C}(\text{O})\text{O}^-$ or a salt thereof, n is an integer from 0 to 12, R_2 is hydrogen or alkyl, R_3 is hydrogen or alkyl, and R_4 is hydrogen
 10 or alkyl.

19. The hemoglobin composition of claim 14 wherein the dithiol is dihydrolipoic acid or a salt thereof.

20. The hemoglobin composition of claim 14 wherein the disulfide is lipoic acid or a salt thereof.

21. The hemoglobin composition of claim 14 wherein the hemoglobin is selected from the group consisting of crosslinked hemoglobin, conjugated hemoglobin, encapsulated hemoglobin, recombinantly-produced hemoglobin, and polymerized hemoglobin.

22. The hemoglobin composition of claim 21 wherein the crosslinked hemoglobin is diaspirin-crosslinked hemoglobin.

23. The hemoglobin composition of claim 14 wherein the hemoglobin composition is in the form of a physiologically acceptable solution for parenteral administration.

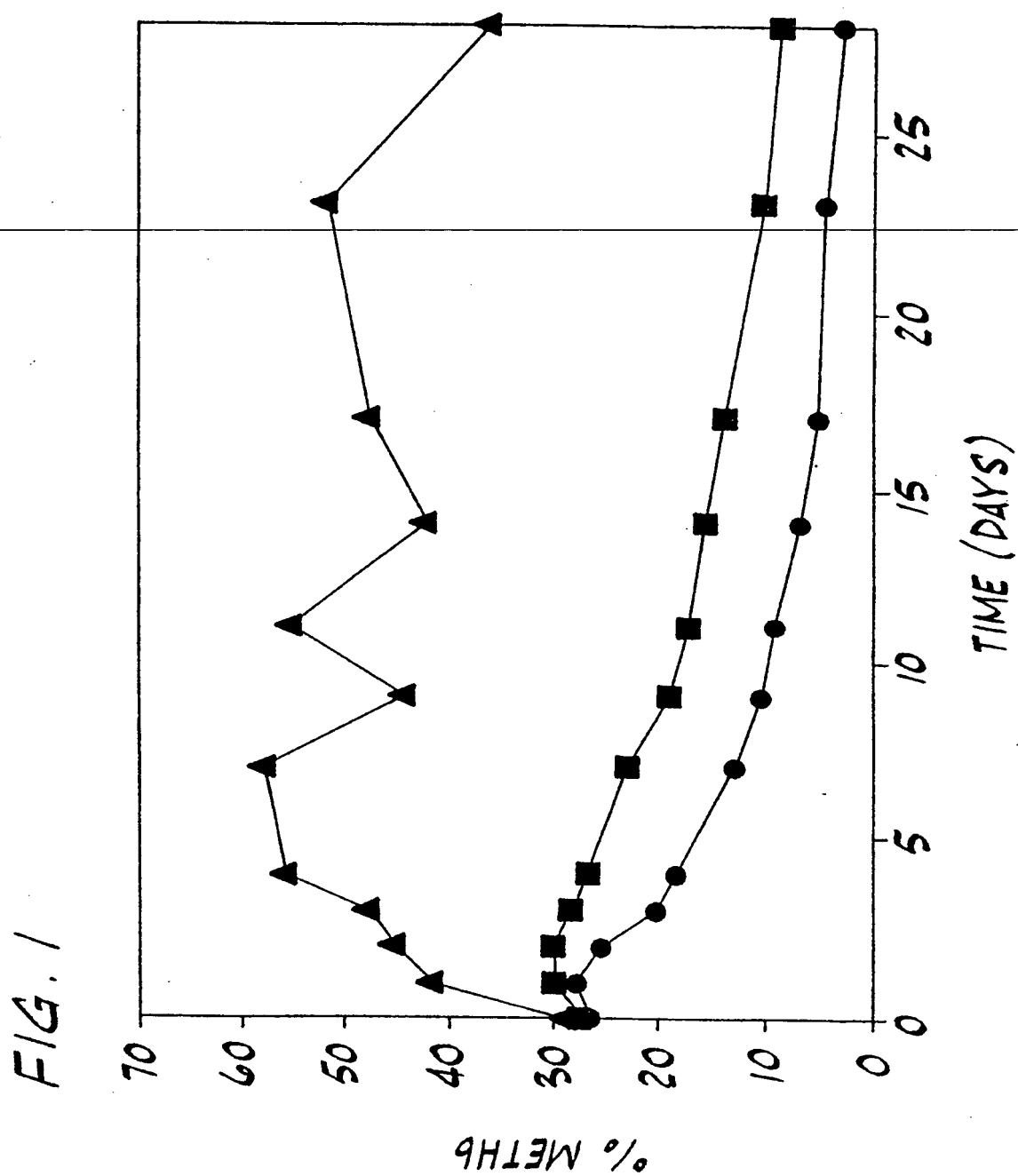
24. The hemoglobin composition of claim 23 wherein the physiologically acceptable solution contains from about 1 g/dl to about 20 g/dl hemoglobin.

25. A process for preparing a hemoglobin composition comprising:
providing a hemoglobin composition containing deoxyhemoglobin and up to about 10% oxyhemoglobin based upon the total hemoglobin content of the hemoglobin composition; and

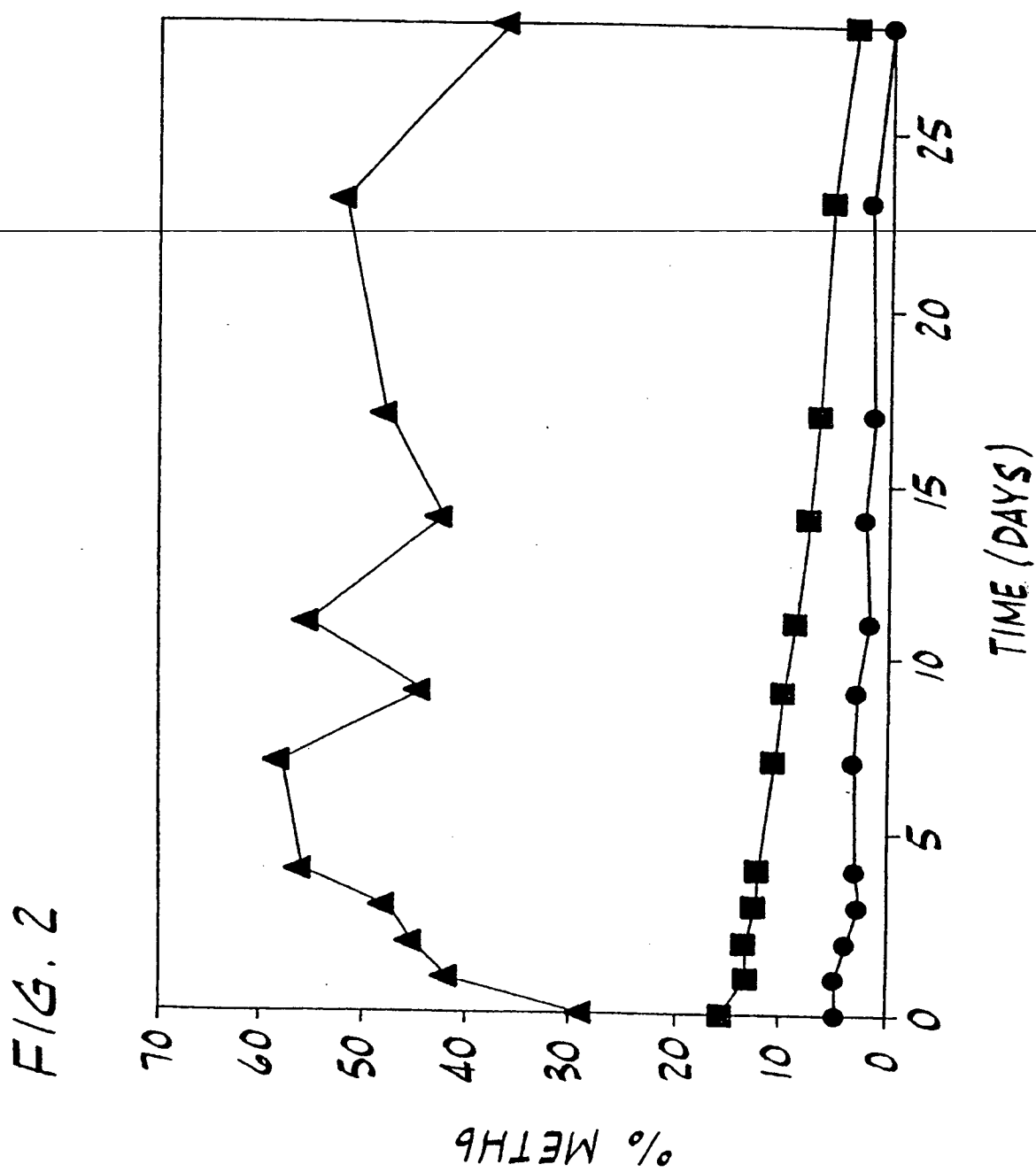
5 admixing a dithiol or a disulfide with the hemoglobin composition.

26. A method for inhibiting degradation of a hemoglobin composition during storage comprising storing a hemoglobin composition containing deoxyhemoglobin and a dithiol or a disulfide in an oxygen-impermeable container at a temperature less than about 40°C.

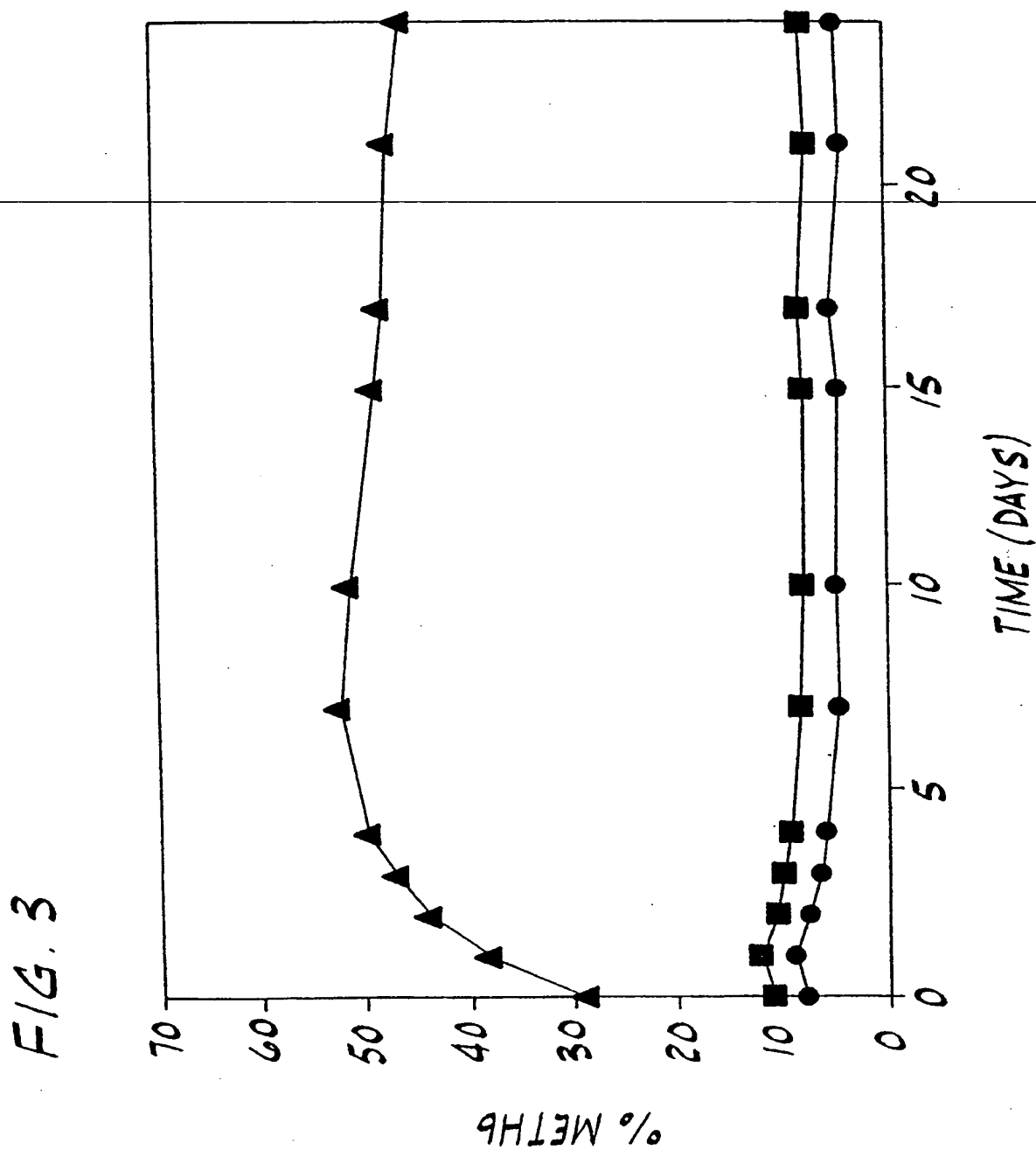
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/19768

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 35/14, 38/16

US CL :424/533; 514/6; 530/385

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/533; 514/6; 530/385

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CA-online, BIOSIS, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,320,965 A (CHIANG) 14 June 1994, see entire document.	1-4, 13-17, 25, 26
Y		1-26
Y	US 5,352,773 A (KANDLER et al.) 04 October 1994, see entire document.	1-26
Y	ROMERO et al. The Reactivity of Thiols and Disulfides with Different Redox States of Myoglobin. Redox and Addition Reactions and Formation of Thiyl Radical Intermediates. Journal of Biological Chemistry. 1992, Vol. 267, No. 3, pages 1680-1688, especially abstract.	1-26

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

05 NOVEMBER 1998

Date of mailing of the international search report

29 DEC 1998

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PCT/US98/19768

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PACKER, L. 'Cell Regulation by Thiol Antioxidants: From Glutathione to Lipoate to Anethole Dithiolethione.' In: Proceedings of the International Symposium on Natural Antioxidants; Mol. Mech. Health Eff. Edited by PACKER et al. Champaign, Ill. AOCS Press 1996, pages 223-235, see entire document.	1-26
A	EP 0 181 033 A1 (AKZO N.V.) 31 October 1984.	1-26
A	WO 85/04407 A1 (BAXTER TRAVENOL LABORATORIES, INC.) 10 October 1985.	1-26